A Simple Chemical Method for the Measurement of Plasma Angiotensin[1] (34322)

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To arrive at a simple method for measuring angiotensin in the blood, we have made use of the competition of endogenous angiotensin and added ^{14}C labeled angiotensin for the angiotensinase in plasma which destroys the octopeptide's pressor activity and leads to liberation of the C-terminal phenylalanine. The validity of the method has been tested with a model system described elsewhere (1). To apply the method to the determination of angiotensin, we had to meet four conditions: there could be no product inhibition; substrate concentrations could not exceed K_m by more than a factor of 10^3, and the test reactions had to be run for a time short enough to leave a virtually constant concentration of angiotensin (substrate). Angiotensin labeled with ^{14}C exclusively on the phenylalanine, but randomly labeled among the phenylalanine's carbon atoms, was used as the added substrate.

Materials and Methods. Theory. The assay was designed to measure angiotensin concentration and angiotensinase activity. By running the reaction at two different substrates concentrations in aliquots of the same plasma, from Formula 2 in Ref. (1),

$$P_m = \frac{S_1 - S_2}{(S_1/P_1) - (S_2/P_2)}. \qquad (1)$$

where $P_m = P_{max}$ in Michaelis-Menten formulas, the maximum amount of product which could be formed during the test reaction by the given amount of enzyme present in the presence of excess substrate; where S_1 = concentration of labeled substrate in first test reaction; S_2 = concentration of labeled substrate in second test reaction; P_1 = concentration of labeled product in first test reaction; P_2 = concentration of labeled product in second test reaction; P_m gave us a measure of angiotensinase activity.

To obtain a measure of renin activity (angiotensin concentration), a formula for $K_m + X$ was derived, where X = concentration of endogenous unlabeled substate (angiotensin),

$$K_m + X = \frac{P_2 - P_1}{(P_1/S_1 - P_2/S_2)}. \qquad (2)$$

While we do not know the K_m for the angiotensinase reaction, the value of $K_m + X$ can be determined. As K_m is constant, this allows an estimation of increments of X.

We also wanted to know how much angiotensin (X_L) remains after the action of angiotensinase. This was arrived at by subtracting the amount of product there would be for the reaction running without added substrate (P),[2] from X, the amount of unlabeled angiotensin present.[3]

$$X_L = (X + K_m) - P_m + K_m\left(\frac{P_m}{X + K_m} - 1\right). \qquad (3)$$

The first two terms are numbers easily obtained as described below, and the last term is a simple function of K_m, a constant.

Assay Method. One-half to 1 ml of heparinized arterial plasma was incubated at 36° with 0.1 to 1 μg (10^{-4} to 10^{-3} μmoles) of angiotensin-1-^{14}C for 20–60 min. The reaction

[1] Supported by Grant HE 10011-02 from the National Heart Institute.

[2] P was derived from Eq. (b) in Ref. (1).

[3] $X_L = X - P$,
$= X + K_m - K_m - P_m + \left(\frac{P_m}{X + K_m}\right)(K_m)$.

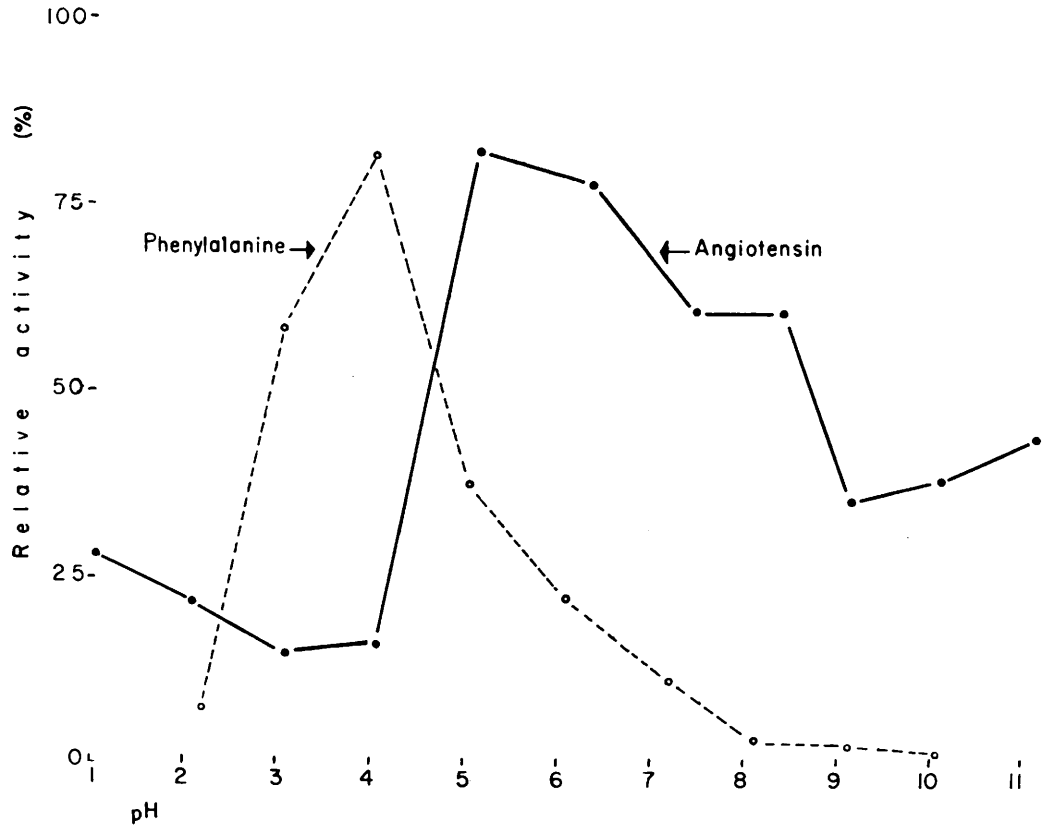

FIG. 1. Relative radioactivity of eluents from two Dowex-50 ion exchange resin columns loaded with phenylalanine-1-^{14}C and angiotensin-^{14}C, respectively. Elutions were made with 0.1 *M* sodium citrate with pH increasing from pH 1.5.

was stopped by adding 25% by volume (of the reaction mixture) of 40% TCA. The precipitated proteins were centrifuged, and supernatant was drawn off. To the supernatant was added 50% by volume of 0.2 *N* NaOH, giving a mixture of pH 4.0. An aliquot of this supernatant was measured for radioactivity, giving a measure of [*S**], or the concentration of radioactive angiotensin at the start of the reaction. Dowex 50 ion exchange resin which had been prewashed with 0.1 *N* Na citrate and adjusted to pH 4.0 was added until the resin occupied 60% of the volume of the mixture. The mixture was shaken twice, and the resin was allowed to settle. The supernatant was sampled for radioactivity, giving a measure of [*P**].

Separate experiments established that phenylalanine was not absorbed onto the Dowex resin when put on a column at pH 4.0. It could be put on the column at pH 3.0, and eluted from the Dowex at pH 4.0. Angiotensin, on the other hand, was absorbed at pH 4.0 but was released sharply at pH 5.0 (see Fig. 1). Furthermore, Dowex resin equilibrated with 0.1 *M* sodium citrate buffer (pH 4.0) was found to absorb 98.5% of 1.29×10^{-8} moles of angiotensin-1-^{14}C in TCA precipitated plasma. Thus the amount of angiotensin involved in the test assay was considered completely absorbable by the resin.

Radioactivity in counts per minute was converted to micrograms of angiotensin by dividing by 27.4 cpm/μg, the specific activity of the radioactive material supplied. The radioactive angiotensin was kindly supplied by Drs. F. A. Kuehl and F. Holly of the Merck Institute.

Results. When a large excess of radioactive

TABLE I. $K_m + X$ Calculated for Angiotensinase Reaction Run with Dog Plasma, and Various Amounts of Added Unlabeled Angiotensin Standard.

Tubes	Added angiotensin, standard (μg)	$K_m + X$ (μg of AT/ml)	X	Error (%)
A	0	2.285		
B	1.25	3.855	1.57	16.0
C	2.5	3.995	1.71	30.0
D	5.0	6.845	4.56	8.0
E	10.0	12.785	10.5	5.0
F	20.0	21.585	19.3	3.5

angiotensin was mixed with arterial dog plasma (120 μmoles of angiotensin/ml of plasma) and incubated for 20 min, the production of radioactive phenylalanine (as measured in aliquots drawn every 5 min for 20 min) was linear with time. This demonstrated that there was no product inhibition under our assay conditions.

The amount of phenylalanine released from angiotension-1-^{14}C in plasma when angiotensinase was completely inhibited was next determined. EDTA, which chelates calcium ions required for plasma angiotensinase activity, completely prevented the release of phenylalanine from 60 μg of angiotensin during incubation with plasma.

Similarly, 200 μg of angiotensin-1-^{14}C incubated with TCA-precipitated plasma yielded no radioactive material which could be eluted from the Dowex 50 resin at pH 4.0.

The ability to measure graded concentrations of unlabeled angiotensin added to the plasma was tested by determining the quantity ($K_m + X$) in plasmas to which increasing amounts of standard angiotensin acid[4] had been added. Radioactive angiotensin was added in two different concentrations to aliquots of plasma. These were incubated with 0.25–20 μg of added unlabeled standard angiotensin. The increments in ($K_m + X$), calculated from formula (2) and the data in Table I, were considered as the calculated amount of endogenous angiotensin. The correspondence between calculated unlabeled angiotensin and the amount actually added is shown in Fig. 2.

[4] Supplied by the National Institute for Medical Research, London, and made for the World Health Organization by CIBA, Basel, Switzerland.

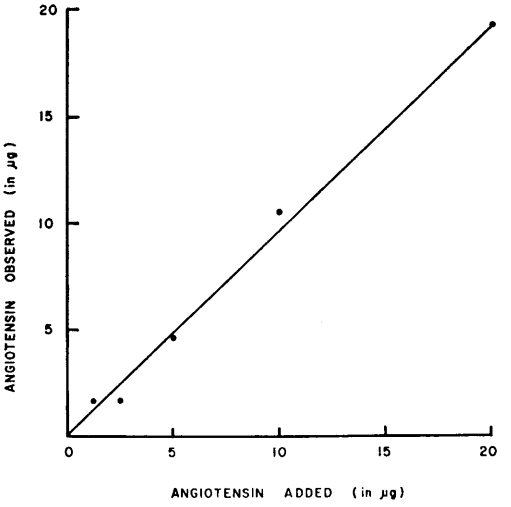

FIG. 2. Plot of angiotensin calculated from assay results versus amount of angiotensin actually added to reaction mixture; the straight line represents perfect correspondence, and was not drawn from the points represented.

By this method angiotensin concentrations were determined in arterial blood from five normal dogs and from seven dogs with unilateral renal artery stenosis or radiation nephritis with various degrees of hypertension. The blood samples were heparinized, centrifuged free of cellular elements, and the plasma kept at 0° until time of assay. Among animals with renal abnormality, angiotensin was positively related to diastolic blood pressure, while among animals with normal kidneys, no correlation of angiotensin with blood pressure was found (Fig. 3) from this data a value of 5 μg of angiotensin II/ml of plasma was taken as the upper limit of normal from these data.

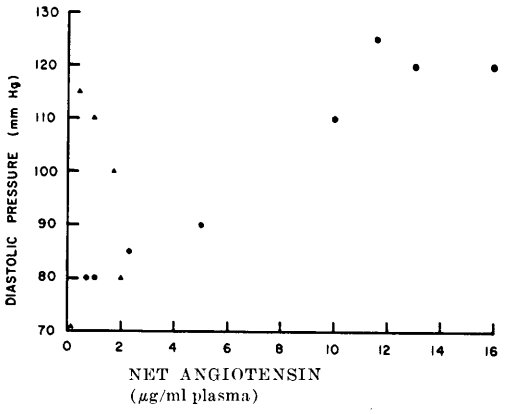

FIG. 3. Net angiotensin accumulation as determined in arterial plasma from seven dogs with unilateral renal artery stenosis or radiation nephritis (circles), and in five dogs without known renal abnormality (triangles), plotted as a function of diastolic arterial blood pressure.

Discussion. The method of measurement of angiotensin presented above can be performed on 6 samples in 2 hr by one technician with standard laboratory equipment. The accuracy of the assay is limited by the accuracy of counting the radioactive materials —and this is determined by the specific activity of the labeled angiotensin used. While our assay was accurate to at least 12%, presumably the accuracy will increase when angiotensin with a higher specific activity is available.

Summary. A simple method for measurement of plasma renin, angiotensin concentration, and angiotensinase activity is presented, based on competition of labeled angiotensin and endogenous angiotensin for the plasma angiotensinase enzyme. Both the accuracy and sensitivity of the method are limited by the specific radioactivity of the labeled angiotensin used.

1. Schwartz, D. T., Britten, J., and Mason, R. C., Proc. Soc. Exptl. Biol. Med., in press.

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